Medium 199[9] enriched by 15% of calf serum was used. Cells were cultured at 37 °C. Medium was changed every 3rd day. Each time it was newly oxygenated. Cultures were continued for 8 days. Slides with monolayers were stained according to the May-Grunwald-Giemsa method to judge their viability.

Beginning from the 4th day of culture, parallel monolayers were submitted to a histochemical test for Δ^5-3β-OH steroid dehydrogenase activity[5,6]. This enzyme participates in the transformation of pregnenolone into progesterone and was observed in corpora lutea in vivo[6,7]. Its presence within cultured cells provided information about their possible hormonal activity.

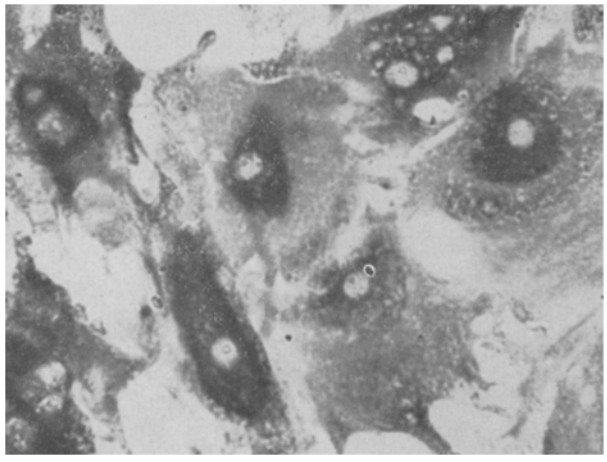

Fig. 6. Distinct Δ^5-3β-OH steroid dehydrogenase activity within porcine luteal cells on the 4th day in culture. ×125.

Both rat and pig corpus luteum cells adapted well to tissue culture conditions, adhesed to the glass early and showed mitotic activity during the culture time. Cells showed typical luteal morphology with no tendency to fibroblastic growth (Figures 1–2). These cells in pig were very similar to separately cultured theca interna cells of the follicle (Figures 3–4). Large and strongly vacuolized cells appeared in 7–8 days cultures (Figure 5). Such cells showed distinct dehydrogenase activity. This would suggest their participation in hormonal synthesis, or aging of cells, if 20α-OH steroid dehydrogenase enzyme would show its activity[8]. Further experiments will decide wheter it is true or not. Cultured corpus luteum cells showed distinct $\Delta^5$3β–OH steroid dehydrogenase activity during investigation time, especially well visible in 4 days culture (Figure 6). The histochemical reaction was both granular and diffuse.

The experiments discussed above are preliminary and further studies are being continued.

Zusammenfassung. Die Fähigkeit der Corpus-luteum-Zellen als Monolayers zu wachsen wurde untersucht. Die von Schwein und Ratte entnommenen Gelbkörper wurden zerkleinert und mit einer 0,25%igen Trypsinlösung in eine Suspension übergeführt. Die Kulturen wurden bis zum 8. Tag beobachtet. Die wachsenden Luteinzellen zeigen die typische Form und auch die ausgeprägte Aktivität der Δ^5-3β-OH Steroiddehydrogenase.

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7 February 1973.

Reconstitution of *Cancer pagurus* Hemocyanin with Copper (II) and Hydroxylamine

Hemocyanin is a non-heme, copper-containing, respiratory protein found in the hemolymph of arthropoda and mollusca. The essential copper cannot be removed from hemocyanin by EDTA or ion-exchange resins. Copper-free hemocyanin can only be prepared without any denaturation of the protein by cyanide treatment[1]. A partial reconstitution of hemocyanin has been achieved by CuC_2^{-1}, Cu_2O and copper (I) amine complex[2]. A practically quantitative reconstitution of functional hemocyanin with copper (I) acetonitrile complex has been reported formerly from these laboratories[3]. In the present communication, a much more simple and quicker method of fixation of copper with copper (II) and hydroxylamine is described.

Copper-free *Cancer pagurus* hemocyanin is prepared by adding dropwise a solution of IM potassium cyanide in 0.01 M hydroxylamine hydrochloride, pH 8.2 (adjusted with acetic acid) to 2% fresh hemocyanin in 0.01 M calcium acetate until the blue protein colour becomes yellowish. This solution is dialyzed for 4 days against 0.05 M potassium cyanide in 0.01 M calcium acetate and 0.01 M hydroxylamine hydrochloride, pH 8.2 (adjusted with acetic acid) at 4 °C. Cyanide is removed by dialysis against acetate buffer (pH 5.7, ionic strength 0.1).

The reconstitution of *Cancer pagurus* hemocyanin is studied through the changes in absorbancy at 335 nm in Beckman DU Spectrophotometer. For the reconstitution, a 2% apohemocyanin solution is treated at pH 5.7, acetate buffer, ionic strength 0.1, for 5 h in presence of nitrogen with 2 equivalents of copper (II) and 50 equivalents of hydroxylamine hydrochloride per mole of copper in native hemocyanin. The reconstituted hemocyanin is dialyzed against EDTA, M/40 in acetate buffer, pH 5.7, ionic strenght 0.1 and afterwards against the acetate buffer alone.

The absorption coefficient, K_a at 335 nm for a protein concentration 1 g/l and a path length of 1 cm, corrected for the absorbance of the protein due to light scattering, is determined. It amounts to 0.231, 0.030, 0.203 for fresh, apo and reconstituted hemocyanin respectively corresponding to 86% reconstitution. Total copper is determined photometrically with 2,9-dimethyl-1,10-phenanthroline at 454 nm in glacial acetic acid in the presence of 0.1% hydroxylamine hydrochloride. The fresh, apo and reconstituted hemocyanins have copper contents of 0.165, 0.001, 0.141% respectively, corresponding to 85% reconstitution.

The reconstituted hemocyanin appears to be identical with the native hemocyanin e.g. the oxygenation-deoxygenation cycles, optical absorption spectra. Copper

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(II) and hydroxylamine thus provides a simple and quicker means for the reconstitution of functional hemocyanin.

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Zusammenfassung. Es wird eine neue und einfache Methode zur Rekonstituierung von Hemocyanin (Apohemocyanin) bei *Cancer pagurus* aus kupferfreiem Hemocyanin beschrieben.

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A Simple Procedure for Detecting Proteins Synthesized in Organ Cultures

At present the study of in-vitro protein synthesis by isolated cells, tissues and organs is widely used for many purposes (e.g., molecular events implicated, effects of drugs, control of hormones). On the other hand, isolation and detection of synthesized proteins generally require complicated methods.

In the course of research on the production of serum proteins by liver explants in vitro, we have developed an immunological procedure for demonstrating the synthesized proteins which involves the utilization as a support medium for immunological analysis of the same nutrient medium used as a nutrient[1]. In such a way the proteins synthesized in the organ cultures, if released, are adsorbed into surrounding medium and so are further detectable by means of immunodiffusion, avoiding their isolation and purification and taking advantage of high sensibility and specificity of antibody-antigen reaction.

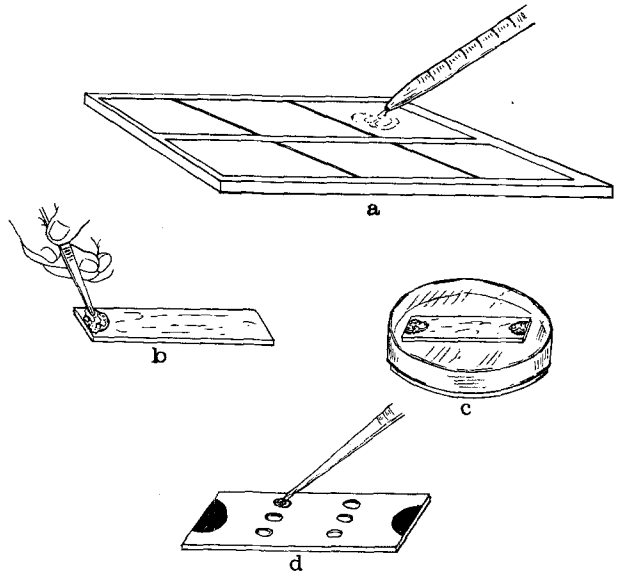

Fig. 1. Some steps of the detection procedure are indicated: a) filling the slides with nutrient medium; b) placing the liver pieces upon the solid media; c) maintaining slides inside petri dishes; d) applying the antiserum into the wells.

To a sterile solution of gelose 1% in Gey fluid kept at 40 °C calf serum and Tyrode's containing sodium merthiolate (0.5 mg/ml; S.I.C., Milano) were added in the following ratios 10:5:1. Sterilyzed glass slides (25 mm × 75 mm; Gelman Instr. Co., Michigan) were covered by means of a 10 ml pipette to obtain a uniform layer of solution (about 4 ml/slide) (Figure 1a).

Livers, removed from 14-day-old chick embryos, were cut and several pieces (each about 1 mm × 1 mm) were placed upon solid media at the extremities of the slides (Figure 1b) and kept in culture in sealed Petri dishes (Figure 1c). At different periods of incubation, the slides were removed, liver explants taken out and processed for histological examination. Wells were then stamped on the slides using a gel punch and the antiserum (anti total chicken serum; Sycco, N.J.) was applied into the wells (Figure 1d).

Immunodiffusion was performed according to Ouchterlony technique[2]. Histological examination demonstrated that under our conditions liver explants underwent a good morphogenesis as previously described[1]. Immunological investigations showed the precipitation lines to be referred to serum proteins (Figure 2).

Riassunto. Viene descritta una tecnica per evidenziare proteine sintetizzate in vitro in espianti d'organo. Tale tecnica è basata sull'impiego del terreno nutrizio solido come substrato per la immunodiffusione.

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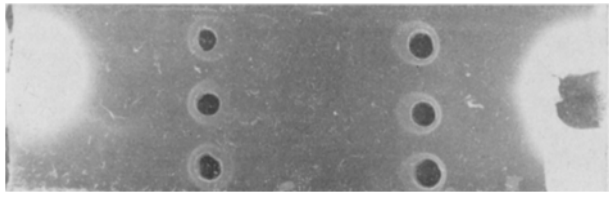

Fig. 2. Outcherlony tests. The precipitation lines are due to reaction between the anti-serum anti total chicken serum protein applied into wells and the serum proteins synthesized by 8 day cultured liver explants and diffused into solid medium.

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[3] These studies were supported by Italian CNR grants No. 69.02110 and No. 70.01069.04